

# Genetic Alterations in Carcinogenesis and Chemoprevention

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Laboratory and clinical studies suggest that genetic change is intrinsically involved in the development of cancer and that this change occurs in humans throughout carcinogenesis, in both early and late stages. Therefore, the quantification of the level of genetic change in human epithelial tissues may serve as a marker for cancer risk. The micronucleus test has been used to quantify the level of site-specific chromosomal breakage occurring in epithelial tissues of individuals at elevated risk for cancer. These studies include individuals exposed to carcinogens, patients who have chromosome-breakage syndromes, and individuals with premalignant lesions. As a counterpart to this approach, the assay has been used to study the suppression of this breakage with chemopreventive agents, some of which occur naturally in the diet. These agents include  $\beta$ -carotene, retinyl palmitate, 13-*cis*-retinoic acid, riboflavin, canthaxanthin, and folic acid. Not all of these agents were effective. The success of the treatment depended on both the agent being used and the population being studied. The results of these studies suggest that successful intervention with chemopreventive agents will depend on tailoring treatment regimens to specific populations. The micronucleus test can be used along with other biological end points to obtain an early indication of the efficacy of chemopreventive agents in altering biological changes associated with carcinogenesis in tissues of high-risk individuals.

## Introduction

In 1982, a National Academy of Science committee examined the epidemiological and laboratory evidence for the role of diet in cancer development (1). This committee issued a report that drew the attention of both the general public and scientists to an association between diets rich in specific compounds and a lower risk of cancer. Intervention to prevent cancer development in high-risk individuals became an intriguing possibility. Many scientists began focusing their research on identifying agents with the potential to inhibit cancer development (collectively termed chemopreventive agents) and, ultimately, on developing approaches for evaluating their effectiveness in reducing cancer incidence in humans.

After a decade of research, scientists are only beginning to understand how and when chemopreventive agents can be used to protect humans from cancer. However, the feasibility of this approach has been demonstrated in several clinical trials, the majority of which have examined the efficacy of chemopreventive agents in reversing premalignant lesions. To date, only two trials have been completed in which cancer itself has been shown to be reduced by chemoprevention in a high-risk population. In both trials, populations were chosen in which cancer devel-

opment was expected to occur at a high rate within a very short time span. Hong and co-workers (2,3) studied patients that had been successfully treated for head and neck squamous cell carcinomas, a group of individuals in which second aerodigestive malignancies occur at a rate of 3-5% per year (2,3). Treatment with 13-*cis*-retinoic acid (cRA) for 12 months resulted in a significant decrease in the incidence of second primary tumors. After 42 months of follow-up, only 6% of cRA-treated patients developed tumors, compared with 27% of patients receiving a placebo. Kraemer and co-workers (4) studied the efficacy of cRA in suppressing the development of carcinomas in xeroderma pigmentosum patients. These patients had a history of multiple cutaneous carcinomas, on average 12.1 tumors per year before treatment. During a 2-year treatment period with cRA, tumor formation was reduced to an average of 2.5 per year. However, when the treatment was discontinued, the tumor frequency increased.

Both of these studies focus attention on an issue of major concern in current efforts to design effective chemoprevention trials. The data indicate that long-term administration of an agent is required for successful intervention because lesions relapse when treatment with the chemopreventive agent is discontinued. Chronic administration of cRA is limited by potential toxicity and side effects. The choice of which agent or combination of agents to use in future regimens is formidable because over a thousand possible chemopreventive agents have been identified. To address this problem, many of the ongoing clinical trials are using batteries of "intermediate markers" to identify

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biological changes that can be used as quick screens for the potential efficacy of chemopreventive agents in specific human populations. Such biological markers could be used to design chemoprevention studies on individuals at earlier stages of carcinogenesis, before the development of any clinical lesion. This paper describes this approach of using intermediate end points as surrogates for clinical lesions and for cancer itself. More specifically, this paper summarizes recent studies on one such biological marker, the micronucleus test on epithelial cells.

## Biological Markers for Intervention

Certain biological events are viewed as intrinsically involved in the development of cancer. These events include the progressive accumulation of alterations to specific genes and a dysregulation of cell proliferation and differentiation controls. Cells no longer mature and senesce, but become immortal. As part of this process of change, promoting agents have been shown to stimulate inflammatory reactions and to elevate rates of cell division.

The last decade has seen the development of a variety of biological assays focused on quantifying these critical events in carcinogenesis (Table 1). Each of these assays is a potential intermediate marker that can be used to screen for chemopreventive agents. Many of these markers have a long history of development in laboratory studies and are only now being modified for human studies. For example, micronuclei are now being evaluated in epithelial tissues by collecting cells as they are exfoliated from the tissue. In many cases, such samples can be obtained noninvasively such as by swabbing the buccal mucosa with a moistened tongue depressor to examine the oral cavity or by centrifuging urine to collect cells from the urogenital tract. Other assays are being modified to be performed on biological fluids such as urine, blood, or saliva or on fine-needle aspirates. In addition, a large effort is being made to increase the information that can be generated from tiny-punch biopsies. Hence, microtechniques are evolving, such as the use of *in situ* hybridization with chromosome-specific probes to quantify aneuploidy in tissues, the use of polymerase chain reactions to identify specific DNA

alterations in cells, and the use of a range of monoclonal antibodies to study minute alterations in both the quantities and types of protein products in a tissue.

Aside from developing intermediate markers and fine-tuning them for use in human populations, laboratory scientists have another large role to play in the area of chemoprevention. Over the years, laboratory research has generated a basic understanding of the diverse mechanisms by which chemopreventive agents act to suppress tumorigenesis (5). Scientists are beginning to classify potential chemopreventive agents by their expected mode of action. Thus, lists have been made of antioxidant scavengers, anti-inflammatory agents, inhibitors of cell proliferation, hormone antagonists, inducers of cell differentiation, modifiers of oncogene expression, and so on. This information can be used to match end points and agents for use in clinical trials of specific populations.

## Micronucleus Test on Epithelial Cells

The micronucleus test is a prime candidate for use as an intermediate marker of chemoprevention because this assay measures an event highly relevant to carcinogenesis. Current indications are that a progressive accumulation of genetic alterations is required for cancer to develop, with these changes occurring throughout carcinogenesis. A chronic increase in the level of genetic damage in a tissue should be associated with an elevation in risk for cancer at that site. Suppression of this damage with a chemopreventive agent could be expected to reduce risk by decreasing the probability of the tissue accumulating all of the genetic changes required for cancer to develop. The use of the micronucleus test as an index of cytogenetic damage is well supported in the literature. Although the test has been most widely used to study genetic damage *in vivo* in preparations from bone marrow and peripheral blood, the assay has the potential for measuring damage at a variety of other sites including the oral mucosa, lung, liver, spleen, urinary bladder, testis, cervix, vagina, esophagus, corneal epithelium, skin, and hair follicles (6).

To date, the micronucleus test has been used to quantify chromosomal breakage in epithelial tissues in over 30

Table 1. Chemoprevention by event.

Event	Potential <i>in vivo</i> biological markers	Examples of chemopreventive agents
Genetic change	Micronuclei, aneuploidy (via <i>in situ</i> hybridization with chromosome-specific probes), chromosomal aberrations (via premature chromosome condensation), DNA adducts	Radical scavengers and antioxidants ( $\beta$ -carotene); Inhibitors of carcinogen bioactivation (oltipraz)
Proliferation	Ornithine decarboxylase activity, antigens for specific proteins (Ki67, PCNA), transcription of genes involved in proliferation ( <i>c-myc</i> , PCNA), and identification of S-phase cells by tritiated thymidine labeling, incorporation of bromodeoxyuridine or flow cytometry	Retinoids, calcium, wheat fiber, DFMO
Inflammation	Indicators of oxidative stress (oxidized DNA bases including thymidine glycol and 8-hydroxyl-2-deoxy-guanosine), induction of proto-oncogenes ( <i>c-fos</i> , <i>c-jun</i> )	Scavengers of reactive oxygen species ( $\beta$ -carotene, ascorbate, $\alpha$ -tocopherol), anti-inflammatory agents (ibuprofen, indomethacin)
Differentiation	Immunohistochemical quantification of specific proteins (e.g., keratins)	13- <i>cis</i> -retinoic acid
Senescence	Histological evidence of apoptosis (programmed cell death)	13- <i>cis</i> -retinoic acid

Abbreviations: PCNA, proliferating cell nuclear antigen; DFMO, difluoromethylornithine.

groups of individuals at risk for cancer (7,8). These groups include individuals with risk factors associated with life-style (e.g., tobacco or alcohol usage, poor diets), genetics (e.g., individuals belonging to cancer-prone syndromes such as xeroderma pigmentosum, ataxia-telangiectasia, or Bloom syndrome), or potential occupational hazards (e.g., pesticides); patients with premalignant lesions (e.g., leukoplakias, dysplasias, metaplasias); and more recently, individuals with pathological conditions associated with chronic inflammation and irritation (e.g., schistosomiasis patients). Together, these studies have included analyses performed on preparations from the oral mucosa, the urinary bladder, the esophagus, the bronchi, and the cervix. In each case, a significant elevation occurred in micronucleus frequencies in samples from the high-risk group compared with those concurrently taken from controls. More importantly, the elevation occurred in the tissue at the site at risk for cancer.

### Micronucleus Test in Chemoprevention Studies

In 1983, we proposed the use of the micronucleus test on epithelial cells as a method of estimating the efficacy of chemoprevention regimens (9). This proposal was based on two observations. First, we had been studying the temporal pattern of micronucleus production in cancer patients receiving radiotherapy to the head and neck region. An increase in micronucleated mucosal cells from the irradiated area occurred within 1 week of the patient commencing treatment. The micronucleus frequency increased throughout treatment, then rapidly declined to values observed in untreated tissues within 1 month of cessation of treatment. This response was thought to reflect the time for cells in the basal cell layer of the epithelium to divide one to two times, followed by maturation and migration to the surface (7). The second observation came from a study concurrently running in the Philippines among chewers of betel quid, a mixture of betel

nut, betel leaf, tobacco, and lime. This chewing habit is strongly associated with an increased risk for oral cancer. An increased frequency of micronucleated cells was observed in exfoliated cell samples obtained by swabbing the oral mucosa of these chewers. It was reasoned that if a chemopreventive agent was to be effective in protecting against genotoxic damage, the response should be quickly apparent as a reduction in micronucleus frequencies in the carcinogen-exposed tissue of such individuals. An intervention trial was initiated in which a group of chewers was given oral supplements of vitamin A (50,000 units retinyl palmitate) and  $\beta$ -carotene (90 mg) twice weekly for 3 months. Although the participants continued chewing, micronucleus frequencies began to decrease within 4 weeks. By 3 months, the average frequency of micronucleated cells had decreased from 4.6 to 0.96%. Frequencies in individuals receiving placebo treatment remained unchanged.

Since this study was published, other intervention trials have used the micronucleus test as an intermediate marker for chemoprevention (Table 2). These trials encompass studies on the oral cavity, urinary bladder, esophagus, and lung and involve several different agents, although  $\beta$ -carotene and vitamin A (as retinyl palmitate or as 13-*cis*-retinoic acid) have been most frequently used. Although these studies are limited in number, they have begun to provide insight into the specificity of action of chemopreventive agents in blocking genetic damage in different populations. For example, several different agents and combinations of agents have been used in trials on betel quid chewers. Trials in which  $\beta$ -carotene, retinyl palmitate, or a combination of these two agents were used all resulted in a significant reduction in micronucleus frequencies. However, when canthaxanthin or folic acid was used, the frequencies remained unchanged. These data suggest that intervention regimens will have to be carefully chosen for specific populations. In further support of this observation are data from studies with reverse smokers in the Philippines. Reverse smokers hold the lit end of the cigarette in their mouths, a habit associated

Table 2. Reduction of genetic damage in high-risk individuals by oral supplementation with chemopreventive agents. (7,8).

Population	Tissue	Treatment	MN frequency	Lesion
Betel quid chewers	Oral cavity (buccal mucosa)	$\beta$ -carotene	Decreased	Partial remission
		Retinyl palmitate	Decreased	Partial remission
		$\beta$ -carotene, retinyl palmitate	Decreased	Partial remission
		Canthaxanthin	No effect	No effect
		Folic acid	No effect	No effect
Reverse smokers	Oral cavity (palate)	$\beta$ -carotene, retinyl palmitate	No effect	No effect
Xeroderma pigmentosum patients	Oral cavity (tongue)	$\beta$ -carotene	Decreased	NE
Snuff-users	Oral cavity (labial mucosa)	$\beta$ -carotene	Decreased	NE
Schistosomiasis patients	Urinary bladder	Praziquantel	Decreased	NE
Individuals with premalignant lesions	Oral cavity	13- <i>cis</i> -retinoic acid	Decreased	Partial remission
	Oral cavity	Riboflavin and retinyl and zinc	No effect	No effect
	Esophagus	Riboflavin and retinyl and zinc acid	Decreased	No effect
	Lung	13- <i>cis</i> -retinoic acid	In progress	In progress

NE, end point was not examined.

with an elevated risk for oral cancer. Although  $\beta$ -carotene/retinyl palmitate supplementation was very effective in protecting betel quid chewers against genetic damage in the oral cavity, this combination had no effect on micronucleus frequencies in reverse smokers.

An issue that needs to be emphasized with respect to the design of future intervention trials using micronuclei is the crucial importance of obtaining as detailed a description as possible of the population undergoing intervention. To date, these groups have involved individuals from many countries, with obvious differences in lifestyle habits, diet, type of carcinogen exposure, socioeconomic status, and many other parameters. In some cases, individuals were chosen to participate in a trial on the basis of a known carcinogen exposure, such as in the choice of betel quid chewers, reverse smokers, and snuff users. In other instances, individuals were chosen because they had premalignant lesions. Whether differences in the stage of carcinogenesis between individuals with and without lesions will play a role in the success of intervention is unknown. In trials in which micronucleus frequencies and lesion response were concurrently monitored, the two end points often showed a parallel response. A decrease in micronucleus frequencies in a tissue was followed somewhat later by at least a partial remission of the premalignant lesions [Table 2, (7)].

Two recently completed studies have focused on other specific characteristics in the trial population. A study on xeroderma pigmentosum patients was the first to show that genetic damage was suppressed by chemoprevention in individuals genetically predisposed to cancer (M. P. Rosin and W. Anwar, unpublished data). This intervention occurred in samples obtained from the dorsal tip of the tongue, a site in these patients at which carcinomas occur at a frequency 20,000 times greater than in controls. The second study involved patients infected with the parasite *Schistosoma haematobium*, an infection strongly associated with cancer of the urinary bladder (10). Several recent reviews have suggested that tissue trauma and inflammatory reactions may play an important role in cancer. Schistosomiasis patients are ideal models for studies of this relationship. Micronucleus frequencies were shown to be significantly elevated in urothelial cells obtained from infected individuals. These frequencies decreased to levels observed in noninfected controls after treatment with the antischistosomal drug praziquantel. This study is important because it suggests that genetic damage could be one mechanism whereby chronic infections could increase cancer risk. Whether a radical scavenger, such as  $\beta$ -carotene, could protect the urothelium from genetic damage during infection is unknown. Such a possibility requires consid-

eration because reinfection is a common problem in schistosomiasis patients. A case could be made for using chemoprevention with a nontoxic component such as  $\beta$ -carotene between praziquantel treatments to protect schistosomal patients from damage to the urothelium due to reinfection.

## Conclusions

Large-scale, long-term clinical trials are required to determine whether responses of specific, intermediate markers to chemoprevention will accurately reflect a reduced incidence of cancer in a supplemented population. However, until such studies are completed, much can be learned about the chemopreventive process by using batteries of end points in studies of high-risk populations. Such intermediate markers may play a major role not only in defining trial populations in more detail, but also in increasing our understanding of the carcinogenic process.

This research was supported by a grant from the National Cancer Institute of Canada with funds from the Canadian Cancer Society.

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